

ADMET & DMPK 6(3) (2018) 242-257; doi: <http://dx.doi.org/10.5599/admet.557>

**ADMET**

Open Access : ISSN : 1848-7718

<http://www.pub.iapchem.org/ojs/index.php/admet/index>

Original scientific paper

## Development and validation of a novel method for simultaneous quantification of enzalutamide, darolutamide and their active metabolites in mice dried blood spots using LC-MS/MS: application to pharmacokinetic study in mice

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Received: June 08, 2018; Revised: July 05, 2018; Available online: July 15, 2018

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### Abstract

A simple, sensitive and rapid assay method has been developed and validated for the estimation of enzalutamide, N-desmethylenzalutamide (active metabolite of enzalutamide), darolutamide and ORM-15341 (active metabolite of darolutamide) on mice dried blood spots (DBS) using liquid chromatography coupled to tandem mass spectrometry with electro spray ionization in the positive-ion mode. The method utilizes liquid extraction of enzalutamide, N-desmethylenzalutamide, darolutamide and ORM-15341 from 3 mm punched disks from DBS cards (spiked or study samples). The extracted sample was chromatographed using an isocratic mobile phase (0.2 % formic acid : acetonitrile; 30:70, v/v) on an Atlantis dC18 column. The total run time was 2.5 min. The MS/MS ion transitions monitored were  $m/z$  465  $\rightarrow$   $m/z$  209,  $m/z$  451  $\rightarrow$   $m/z$  195,  $m/z$  399  $\rightarrow$   $m/z$  178,  $m/z$  397  $\rightarrow$   $m/z$  194 and  $m/z$  481  $\rightarrow$   $m/z$  453 for enzalutamide, N-desmethylenzalutamide, darolutamide, ORM-15341 and the IS (apalutamide- $d_3$ ), respectively. Method validation was performed as per regulatory guideline. The assay had a good linearity over the range of 0.93-2000 ng/mL. The intra- and inter-batch accuracy and precision (%RE & RSD) across quality controls met the acceptance criteria for all the analytes. Stability studies showed that all the analytes were stable on DBS cards for one month. This novel method has been applied to analyze the DBS samples of enzalutamide, N-desmethylenzalutamide, darolutamide and ORM-15341 obtained from a pharmacokinetic study in mice.

### Keywords

Enzalutamide; N-desmethylenzalutamide; darolutamide; ORM-15341; dried blood spot; LC-MS/MS; method validation; mice; pharmacokinetics

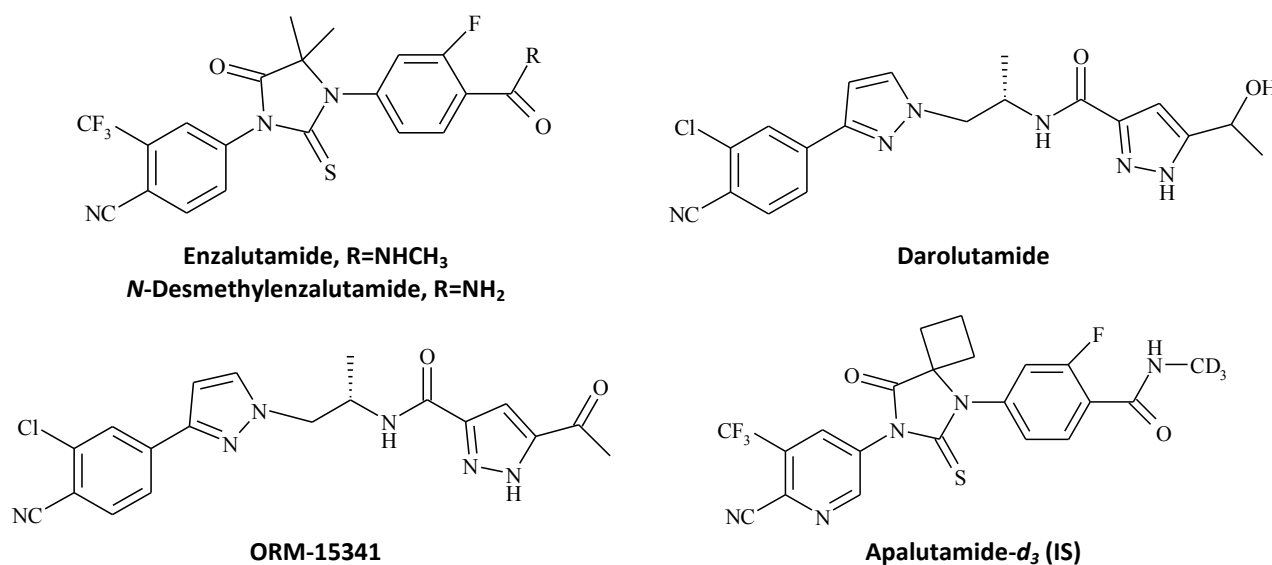
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### Introduction

Despite therapeutic advances, prostate adenocarcinoma is the highly prevalent cancer in men and responsible for 20 % of cancer related deaths in the Western world [1]. Initial approach to cure the localized prostate cancer is through radiation therapy or surgical castration or treatment with first-generation anti-androgens (flutamide, nilutamide, bicalutamide etc.) [2]. However, despite an initial response, most patients develop a most aggressive form of disease called metastatic castration-resistant prostate cancer (mCRPC) that is associated with tumor progression and survival less than 18-24 months [3]. Consequently, in recent years, new therapy options for mCRPC with different mechanisms of action have

become available like targeting androgen receptor signalling such as CYP17A1 inhibitor (abiraterone acetate) [4] and a second-generation androgen receptor antagonist (enzalutamide, apalutamide and darolutamide) [5-7]. Treatment with these drugs showed improved overall survival and quality of life in mCRPC patients.

Enzalutamide (Xtandi<sup>®</sup>; Fig. 1) was recently approved for the treatment of mCRPC [5,8]. Enzalutamide is metabolized by CYP3A4 and 2C8 to produce an active circulatory metabolite, *N*-desmethylenzalutamide (Fig. 1), which has similar in vitro potency to enzalutamide [9,10]. Darolutamide (ODM-201; Fig. 1) is a novel, orally active, second-generation non-steroidal anti-androgen. It is an equi-mixture of two pharmacologically active diastereomers namely ORM-16497 and ORM-16555. Darolutamide undergoes extensive Phase-I metabolism and produces circulatory metabolite i.e., ORM-15341 (Fig. 1). ORM-16497, ORM-16555 and ORM-15341 are fully antagonist against bicalutamide, hydroxyflutamide, enzalutamide and ARN-509 mutants [11,12]. Unlike enzalutamide and apalutamide, penetration of darolutamide and ORM-15341 into brain is negligible (in preclinical species) suggests that low risk of causing seizures in patients [7]. In clinic, darolutamide was tolerated up to 1800 mg and no dose-limiting toxic effects were reported. It was reported that ORM-15341 plasma concentrations were higher than sum of ORM-16497 and ORM-16555 plasma concentrations [13]. Currently Phase-III clinical trials are being conducted with darolutamide in non-mCRPC patients globally [7].



**Figure 1.** Structural representation of enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and apalutamide-*d*<sub>3</sub> (IS)

In contemporary literature few LC-MS/MS bioanalytical methods were reported for quantification of enzalutamide alone [14] or along with its metabolites [15,16] and other anti-androgens [17-19] or anticancer drugs [20] in various biological matrices (preclinical species plasma/tissues and human plasma). Similarly darolutamide and ORM-15341 quantification in mice plasma was reported post-administration of darolutamide [21] and along with other second-generation non-steroidal anti-androgens (enzalutamide, apalutamide) [20] using conventional achiral columns on LC-MS/MS. Apart from these achiral methods, two chiral LC-MS/MS methods for quantification darolutamide diastereomers alone [22] or along with its metabolite [23] were reported in recent times.

Traditional bioanalysis of samples from preclinical species and clinical trials relies on collection of blood or its products (serum and plasma). Collection of blood in a preclinical set up especially with rodents, pediatric cases and new-borns is a challenge and not possible to draw blood samples at multiple time

points. This will force researcher to adopt sparse sampling, which limits the researcher to study the pharmacokinetics from individual animal/neonate. Traditional samples are normally shipped on dry ice from one place to other, which incurs shipping and handling costs. Over the wet sampling (blood/plasma/serum) techniques, dried blood spots (DBS) method offers several advantages like reduced blood/plasma/serum volume per time point, ease and safety in handling, potential cost saving in storage and shipment at ambient room temperature, less sample processing time and increase in throughput etc. [24].

To the best of our knowledge, simultaneous method for the determination of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 on mice DBS has not been reported in the literature. For this purpose, the present study is aimed to develop and validate a simple and rapid LC-MS/MS method for simultaneous quantification of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 on mice DBS without compromising on sensitivity. The validated method was successfully used in a mice pharmacokinetic study following intravenous administration of enzalutamide and darolutamide at 1.0 mg/kg dose.

## Materials and methods

### Materials

Enzalutamide (purity: >98.3 %) and *N*-desmethylenzalutamide (purity: >99.6 %) were purchased from BioOrganics, Bangalore, India. Darolutamide (purity: 98 %) was procured from Angene International Limited, China. ORM-15341 (purity: 99.4 %) and apalutamide-*d*<sub>3</sub> (purity: 99.5 %; Fig. 1) were synthesized by the Medicinal chemistry group, Jubilant Biosys (Bangalore, India) using literature information [25,26] and characterized using chromatographic (HPLC, LC-MS/MS) and spectral techniques (IR, UV, Mass, <sup>1</sup>H and <sup>13</sup>C-NMR) by the Analytical research group, Jubilant Biosys. HPLC-grade acetonitrile, methanol, methyl tert-butyl ether (TBME) and ethyl acetate were purchased from Rankem, New Delhi, India. Whatman FTA DMPK card C DBS cards were purchased from GE, Bangalore. Silica gel sachets and sealable plastic bags for the storage of DBS cards were purchased from local market.

### Instrumentation and chromatographic conditions

A Shimadzu VP (Shimadzu, Japan) LC system equipped with degasser (G1379A), quaternary pump (10ADvp), column oven (CTO-10ASvp), auto-sampler (SIL-HTC) along with system controller (SCL-10Avp) was used to inject 5.0 µL aliquots of the processed samples on an Atlantis dC18 column (50 x 4.6 mm, 3 µm; Waters, Milford, MA, USA), which was kept at ambient temperature (24 ± 1 °C). The isocratic mobile phase, a mixture of 0.2 % formic acid : acetonitrile (30:70, v/v) was filtered through a 0.45 µm membrane filter (XI5522050) (Millipore, USA or equivalent) and then degassed ultrasonically for 5 min was delivered at a flow rate of 0.8 mL/min (with splitter 50 %) into the mass spectrometer electro spray ionization chamber.

Quantitation was achieved by MS/MS detection in positive ion mode for the analytes and the IS using Sciex (Foster City, CA, USA) API-6500 mass spectrometer, equipped with a Turboionspray™ interface at 500 °C and 5500 V ion spray voltage. The common parameters, i.e. curtain gas (CUR), nebulizer gas (GS1), auxiliary gas (GS2) and collision gas (CAD) were set at 55, 55, 65 and 10 psi, respectively. The compounds parameters, i.e. declustering potential (DP), collision energy (CE), collision exit potential (CXP) and entrance potential (EP) for the analytes and the IS are shown in Table 1. The dwell time was 100 msec. Quantitation of enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS was achieved by

monitoring the precursor Q1 → Q3 ions at  $m/z$  465→209, 451→195, 399→178, 397→194 and 481→453, respectively. Both Q1 and Q3 quadruples were set on unit resolution. The analytical data was processed by Analyst software (version 1.6.2).

**Table 1.** Optimized mass spectrometry parameters for analytes and the IS

Analyte	in Volts			
	DP	EP	CE	CXP
Enzalutamide	80	10	41	12
<i>N</i> -Desmethyl-enzalutamide	80	10	41	12
Darolutamide	91	10	31	30
ORM-15341	60	10	27	12
Apalutamide- $d_3$ (IS)	100	10	33	12

DP: declustering potential; EP: entrance potential; CE: collision energy and CXP: collision cell exit potential

#### Preparation of stock and standard solutions

Primary stock solutions of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 for preparation of calibration curve (CC) and quality control (QC) samples were prepared from separate weighing. Individual primary stock solution of all the analytes at 200 µg/mL were prepared in DMSO:methanol (0.2:99.8, v/v). Similarly the primary stock solution of the IS (200 µg/mL) was prepared in methanol. The primary stock solutions of enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS were stored at -20 °C, which were found to be stable for one month. The primary stock solutions of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 were successively diluted in DMSO:methanol (0.2:99.8, v/v) to prepare secondary stock and working stock solutions to prepare CC and QC samples. Working stock solutions were stored approximately at 4 °C for 30 days and found to be stable. A working stock of the IS solution (25 ng/mL) was prepared in methanol. Samples for the determination of precision and accuracy were prepared by spiking control mice blood in bulk with mixed working stock solution of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 at appropriate concentrations 0.93 ng/mL (lower limit of quantitation quality control, LLOQ QC), 2.80 ng/mL (low quality control, LQC), 853 ng/mL (medium quality control, MQC) and 1493 ng/mL (high quality control, HQC). DBS cards were prepared (as mentioned in Blood spotting section) at each QC level and were stored at -80 ± 10 °C until analysis.

#### Blood spotting

The DBS cards were prepared from freshly drawn whole blood, harvested on the same day by spotting 25 µL of the respective spiked CC/QC or whole blood from the enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 treated mouse onto sampling paper using a calibrated pipette. The samples were left to dry at room temperature least 3 h before storing at controlled room temperature (24 ± 1 °C) until analysis.

#### Recovery

The efficiency of enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS extraction from DBS samples was determined by comparing the responses of the analyte extracted from replicate QC samples (n = 6) with the response of analyte from neat standards at equivalent concentrations by liquid extraction process. Recovery of enzalutamide, *N*-desmethylenzalutamide, darolutamide and

ORM-15341 was determined at LQC (2.80 ng/mL) and HQC (1493 ng/mL) concentrations. Recovery of the IS was determined at a single concentration of 25 ng/mL. Samples were prepared by punching a 3 mm diameter disk from the centre of the DBS using a hole puncher (Harri-Micro-Punch<sup>®</sup>, 3 mm circle) into a 2.0 mL microcentrifuge tube using the 200  $\mu$ L of extraction solvents viz., TBME, ethyl acetate, methanol, acetonitrile, methanol/acetonitrile:water (in different ratios).

#### *Sample preparation*

To each DBS card (3 mm) in a glass tube, 200  $\mu$ L of water was added vortex mixed for 10 min and sonicated for 10 min at room temperature. Post sonication 200  $\mu$ L of acetonitrile enriched with the IS (25 ng/mL) was added vortex mixed for 10 min followed by centrifugation 10 min. Clear supernatant (200  $\mu$ L) was separated and transferred into a HPLC vial for injection and 5.0  $\mu$ L was injected onto LC-MS/MS system.

#### *Method validation*

The validation experiments were performed in accordance with the US Food and Drug Administration guideline [27].

#### Selectivity

Selectivity of the method was determined by determining the presence of interfering peaks from six individual drug-free mice DBS samples at the retention times of enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS.

#### Limit of quantification and carry over

The LLOQ was determined as the concentration that has a precision of <20 % of the relative standard deviation and accuracy between 80-120 % of the theoretical value. The auto-injector carryover was determined by injecting the highest calibration standard, followed by injection of blank samples. The response of the blanks was then compared to that of the LLOQ.

#### Calibration curve

Calibration samples (for all the analytes) were prepared on each validation day. Peak area ratios of each analyte to that of IS were used for all calculations. A least squares linear regression (1/X<sup>2</sup> weighting factor) of eight non-zero samples was used to define the calibration curve.

#### Precision and accuracy

The precision and accuracy of the method were evaluated by measuring the four QC samples (LLOQ QC, LQC, MQC and HQC), which were prepared on each validation day (n = 6 each). Inter-day precision was assessed on three separate days. Inter- and intra-day precisions were determined by calculating percent relative standard deviation (%RSD) that should be <15 % for all the QC levels except for LLOQ QC where it should be <20 %. The inter- and intra-day accuracy expressed as percent relative error (%RE) was calculated by comparing the measured concentration with the nominal value and deviation was limited within  $\pm 15$  % except for LLOQ QC where it should be <20 %.

#### Matrix effect

Matrix effect for enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 was assessed by comparing each analyte mean peak areas at LQC and HQC concentration after with the mean peak areas for post extracted DBS samples spiked with analyte at equivalent concentrations. Matrix effect for the IS

was assessed at single concentration (25 ng/mL).

### Stability

The stability of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 was assessed at LQC and HQC levels in six replicates under all storage conditions. Freeze-thaw stability was performed following three freeze-thaw cycles was evaluated (DBS cards were stored in  $-80 \pm 10$  °C between freeze/thaw cycles). Short-term temperature stability was assessed by analyzing samples that had been kept at ambient temperature ( $25 \pm 1$  °C) for 7 days. Long-term stability was performed by analyzing samples that had been stored at  $-80 \pm 10$  °C for 30 days. The stability of enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS in the injection solvent was determined periodically by injecting replicate preparations of processed DBS samples for up to 24 h (in the auto-sampler at 5 °C) after the initial injection.

### Dilution effect

To evaluate the effect of dilution over the calibration range, the accuracy and precision of dilution control samples at 10,000 ng/mL ( $n = 6$ ; 5 times of the ULOQ) were assessed by performing a 10-fold dilution.

### *Influence of hematocrit*

Hematocrit (Hct) has a significant effect on the viscosity of the blood, which influences the flux and diffusion properties of the blood through DBS card used for sample collection. It can directly affect the accuracy of the analysis in DBS samples. Hct is normally about 43-48 for Balb/C mice (in house data). The effect of the Hct on analytical performance was investigated by measuring LQCs and HQCs spiked in six different sources of blood adjusted with plasma to obtain values at 25, 35 and 45 % haematocrit that were analysed with calibrators prepared in blood at standard fixed 40 % Hct. The relative error of  $\pm 15$  % and precision of  $\leq 15$  % was considered acceptable. Hematocrits were measured using Mindray BC-5000Vet.

### *Pharmacokinetic study*

All the animal experiments were approved by Institutional Animal Ethical Committee (IAEC/JDC/2017/133). Male Balb/C mice ( $n = 12$ ) purchased from Vivo Biotech, Hyderabad were housed in Jubilant Biosys animal house facility in a temperature ( $22 \pm 1$  °C) and humidity (30-70 %) controlled room (15 air changes/h) with a 12:12 h light:dark cycles, had free access to rodent feed (Altromin Spezialfutter GmbH & Co. KG., Im Seelenkamp 20, D-32791, Lage, Germany) and water for one week before using for experimental purpose. Following ~4 h fast (during the fasting period animals had free access to water) mice (25-32 g) received enzalutamide and darolutamide intravenously [5 % DMSO, 5 % Solutol:absolute alcohol (1:1, v/v) and 90 % of normal saline; strength: 0.1 mg/mL; dose volume: 10 mL/kg] as a cassette dose at 1.0 mg/kg. Post-dosing serial blood samples (25  $\mu$ L, sparse sampling was done and at each time point three mice were used for blood sampling) were collected at regular intervals using micropipettes (Microcaps®; catalogue number: 1-000-0500) through tail vein into polypropylene tubes containing K2.EDTA solution as an anti-coagulant 0.12, 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144 and 168 h and spotted onto the cards and air dried at room temperature for 3 h before storing at controlled room temperature (25 °C) until analysis. Animals were allowed to eat feed 2 h post-dose of enzalutamide and darolutamide.

The criteria for acceptance of the analytical runs encompassed the following: (i) 67 % of the QC samples accuracy must be within 85-115 % of the nominal concentration (ii) not less than 50 % at each QC concentration level must meet the acceptance criteria [27]. Blood concentration versus time data of



enzalutamide, darolutamide and ORM-15341 was analyzed by non-compartmental method using Phoenix WinNonlin Version 7.0.

## Results

### *MS/MS conditions and chromatography*

The optimum LC-MS/MS mobile phase was determined through several trials to achieve good resolution and symmetric peak shapes for the analytes and the IS in short run time. To obtain good separation, resolution and peak shape for enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS isocratic elution was used at a flow rate 0.8 mL/min. Among the several commercial columns (Inertsil, Atlantis, Kromasil, Hypersil etc.) tested, an Atlantis dC18 column (50 x 4.6 mm, 5  $\mu$ m) was selected because it provided symmetric peaks and baseline separation of enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS within 2.5 min run time. The MS/MS ion transitions monitored were  $m/z$  465 $\rightarrow$ 209,  $m/z$  451 $\rightarrow$ 195,  $m/z$  399 $\rightarrow$ 178,  $m/z$  397 $\rightarrow$ 194 and  $m/z$  481 $\rightarrow$ 453 for enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS, respectively for quantitation. The retention time of enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS was 1.32, 1.23, 1.01, 1.12 and 1.43 min, respectively. The total run time is 2.5 min (Fig. 2a-d).

### *Method validation*

#### Selectivity

As shown in Figures 2a-d no significant interferences in the blank DBS traces were found from endogenous components in drug-free mice blood at the retention times of the enzalutamide, *N*-desmethylenzalutamide, darolutamide, ORM-15341 and the IS indicating that the method is selective.

#### Sensitivity

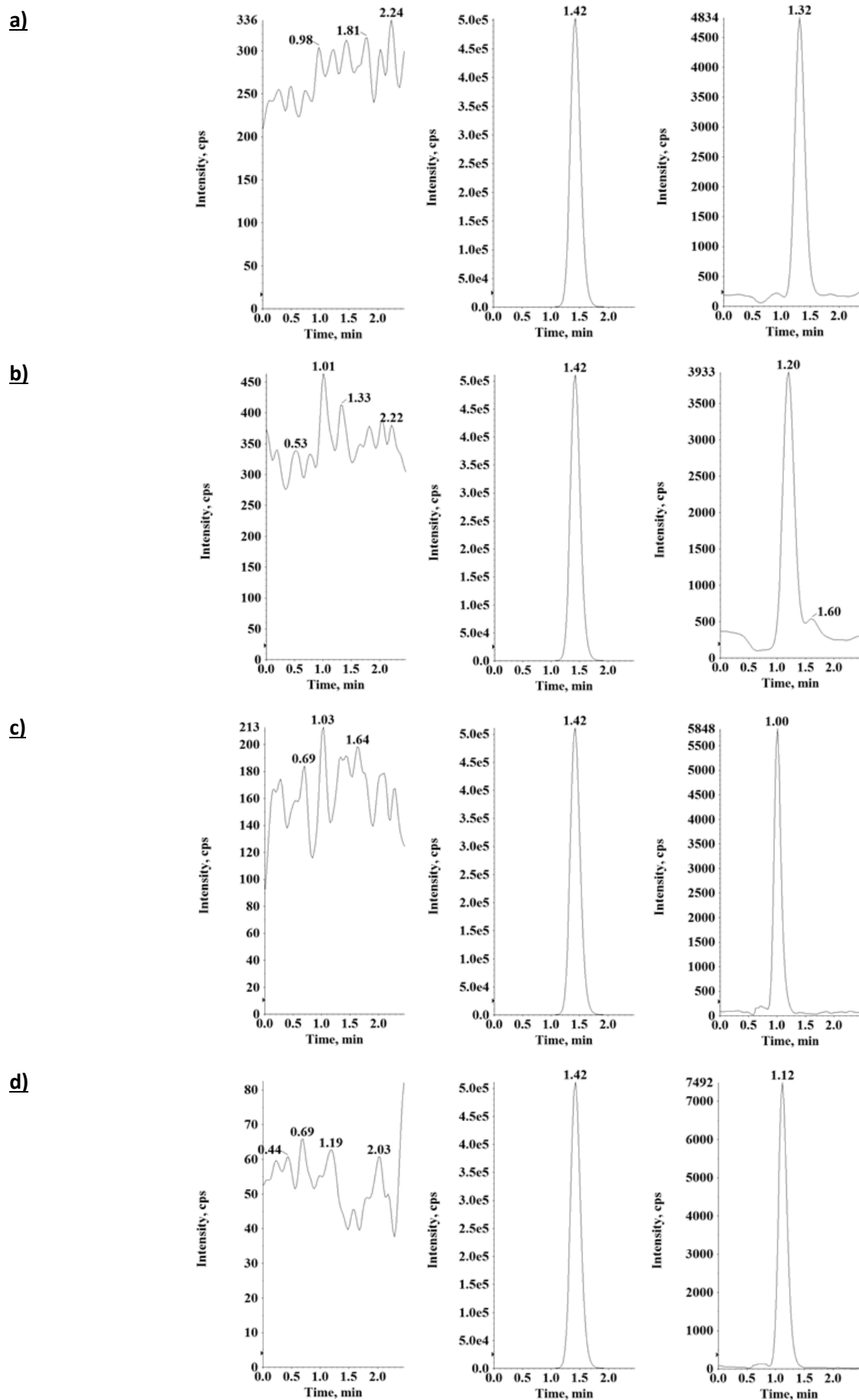
The lowest limit of reliable quantification for the analyte was set at the concentration of the LLOQ. The precision and accuracy at LLOQ concentration were found to be 7.00 and 103 % for enzalutamide; 3.70 and 98.0 % for *N*-desmethylenzalutamide; 5.25 and 95.2 % for darolutamide and 2.85 and 103 % for ORM-15341. There was no carry-over produced by the highest calibration sample on the following injected blank DBS extract sample.

#### Recovery

The recovery (mean  $\pm$  S.D) for enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 (at LQC, MQC and HQC) is presented in Table 2. Recovery for the IS was  $85.4 \pm 5.89$  %.

#### Matrix effect

Six different lots of DBS samples, spiked with analytes concentration levels at LQC and HQC levels were analyzed. The results have shown that the precision and accuracy for analyzed samples were within acceptance range (Table 2). Overall it was found that there is no impact on the ionization of analytes and the IS. Matrix effect for the IS was  $105 \pm 1.25$  %.



**Figure 2.** Typical MRM chromatograms mice blank DBS (left panel), IS (middle panel) and LLOQ (right panel) for **(a)** enzalutamide **(b)** *N*-desmethylenzalutamide **(c)** darolutamide and **(d)** ORM-15341



**Table 2.** Recovery and matrix data for enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 quality controls on DBS cards

	Mean recovery, % Mean $\pm$ SD (n=4)	Mean absolute matrix effect, % Mean $\pm$ SD (n=4)
<b>LQC (2.80 ng/mL)</b>		
Enzalutamide	84.4 $\pm$ 11.0	99.2 $\pm$ 5.85
<i>N</i> -Desmethylenzalutamide	78.0 $\pm$ 7.83	101 $\pm$ 8.58
Darolutamide	47.2 $\pm$ 4.55	88.3 $\pm$ 2.34
ORM-15341	79.7 $\pm$ 15.4	95.8 $\pm$ 6.23
<b>HQC (1493 ng/mL)</b>		
Enzalutamide	78.6 $\pm$ 4.50	95.3 $\pm$ 12.0
<i>N</i> -Desmethylenzalutamide	76.7 $\pm$ 3.75	108 $\pm$ 5.68
Darolutamide	50.3 $\pm$ 1.99	85.2 $\pm$ 1.35
ORM-15341	77.1 $\pm$ 3.32	98.6 $\pm$ 2.38

SD: standard deviation

### Calibration curve

The blood calibration curve was constructed using eight calibration standards (viz., 0.93-2000 ng/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. Calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the  $y = mx + c$  using weighing factor ( $1/X^2$ ). The average slope and intercept values were found to be 0.005358 and 0.002326; 0.005078 and -0.000221; 0.00682 and 0.000485; 0.00967 and 0.000272 for enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341, respectively. The average regression ( $n = 4$ ) was found to be  $> 0.99$  for all the analytes. The lowest concentration with the RSD  $< 20\%$  was taken as LLOQ and was found to be 0.93 ng/mL. The % accuracy observed for the mean of back-calculated concentrations for four calibration curves was within 86.8-114; 85.0-108; 87.1-110 and 90.9-103; while the precision (% CV) values ranged from 4.42-10.3; 5.31-9.34; 1.60-11.5 and 1.12-9.62 for enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341, respectively.

### Accuracy and precision

Accuracy and precision data for intra- and inter-day DBS samples for enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 are presented in Table 3. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

### Stability

The measured concentrations for enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 at 2.80 and 1493 ng/mL were within 85-115 % of RE and  $< 15\%$  RSD in a battery of stability tests viz., in-injector (24 h), at room temperature for 7 days, repeated three freeze/thaw cycles and freezer stability at  $-80 \pm 10^\circ\text{C}$  for at least for 30 days (Table 4).

### Dilution effect

The accuracy of the nominal concentration of the diluted DBS samples were within 5.28 % and the precision was 8.56 % for all the analytes, which show the ability to dilute samples up to a dilution factor of ten in a linear fashion.

**Table 3.** Intra- and inter-day precision and accuracy determination for determination of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 quality controls on DBS cards

Parameters	Enzalutamide	<i>N</i> -desmethyl-enzalutamide	Darolutamide	ORM-15341
<b>Intra-day</b>				
<b>LLOQ QC (0.93 ng/mL)</b>				
Precision (% RSD)	6.09	2.08	9.36	2.68
Accuracy (%RE)	0.97	0.92	1.02	0.96
<b>LQC (2.80 ng/mL)</b>				
Precision (% RSD)	3.24	0.18	10.2	11.7
Accuracy (%RE)	0.90	0.96	1.09	0.96
<b>MQC (853 ng/mL)</b>				
Precision (% RSD)	6.53	3.45	5.17	4.73
Accuracy (%RE)	1.04	1.11	0.97	1.14
<b>HQC (1493 ng/mL)</b>				
Precision (% RSD)	0.90	3.92	5.26	12.0
Accuracy (%RE)	0.99	1.00	0.86	1.13
<b>Inter-day</b>				
<b>LLOQ QC (0.93 ng/mL)</b>				
Precision (% RSD)	8.01	5.01	6.54	1.02
Accuracy (%RE)	1.00	1.07	1.05	1.06
<b>LQC (2.80 ng/mL)</b>				
Precision (% RSD)	7.54	3.29	10.8	5.25
Accuracy (%RE)	0.96	1.04	0.96	1.02
<b>MQC (853 ng/mL)</b>				
Precision (% RSD)	2.38	2.73	8.58	10.8
Accuracy (%RE)	1.02	1.12	1.12	1.14
<b>HQC (1493 ng/mL)</b>				
Precision (% RSD)	2.18	1.58	11.2	9.28
Accuracy (%RE)	1.04	0.99	1.00	1.14

% RSD: relative standard deviation (SD x 100/mean)

% RE: relative error (measured value/actual value)

### Hematocrit effect

The measured enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 concentrations were compared with the results obtained from DBS samples are given in Table 5. The % difference was calculated by subtracting the % relative error of Hct 25 and 45 % from % relative error of 40 % Hct which was taken as standard Hct value. No significant impact of Hct on accuracy was observed for all the analytes.

**Table 4.** Stability data of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 quality controls on DBS cards

Parameters	Enzalutamide	<i>N</i> -desmethyl-enzalutamide	Darolutamide	ORM-15341
<b>Room temperature (7 days)</b>				
<b>LQC (2.80 ng/mL)</b>				
Precision (% RSD)	2.58	7.58	5.98	7.68
Accuracy (%RE)	0.91	0.86	0.95	0.98
<b>HQC (1493 ng/mL)</b>				
Precision (% RSD)	5.84	5.68	3.56	4.89
Accuracy (%RE)	1.02	0.95	0.98	0.86
<b>In-injector (24 h)</b>				
<b>LQC (2.80 ng/mL)</b>				
Precision (% RSD)	6.78	0.73	7.04	4.60
Accuracy (%RE)	0.87	0.85	0.90	0.94
<b>HQC (1493 ng/mL)</b>				
Precision (% RSD)	8.58	10.3	9.08	11.0
Accuracy (%RE)	0.95	0.86	1.00	0.87
<b>3 F/T cycle</b>				
<b>LQC (2.80 ng/mL)</b>				
Precision (% RSD)	5.03	6.60	8.65	5.68
Accuracy (%RE)	1.10	0.99	0.95	0.88
<b>HQC (1493 ng/mL)</b>				
Precision (% RSD)	6.47	11.6	5.28	11.7
Accuracy (%RE)	1.03	0.94	0.94	0.86
<b>Long-term stability at -80°C (30 days)</b>				
<b>LQC (2.80 ng/mL)</b>				
Precision (% RSD)	4.77	9.21	10.2	6.60
Accuracy (%RE)	1.01	1.02	0.91	0.89
<b>HQC (1493 ng/mL)</b>				
Precision (% RSD)	4.03	8.81	10.2	4.85
Accuracy (%RE)	1.04	0.99	0.87	0.85

% RSD: relative standard deviation (SD x 100/mean)

% RE: relative error (measured value/actual value)

### Pharmacokinetic study

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the pharmacokinetics of enzalutamide, *N*-desmethylenzalutamide (released from enzalutamide), darolutamide and ORM-15341 (released from darolutamide) in blood following cassette dose intravenous administration at 1.0 mg/kg dose of enzalutamide and darolutamide to male BalbC mice. Fig. 3a shows mean blood concentration-time profile for enzalutamide and it was quantified till 168 h post-dosing by intravenous route. However the blood concentrations for *N*-desmethylenzalutamide were seen just above LLOQ at 4, 8, 24 and 48 h (ranged between 1.23-4.30 ng/mL) and below LLOQ at remaining time points hence

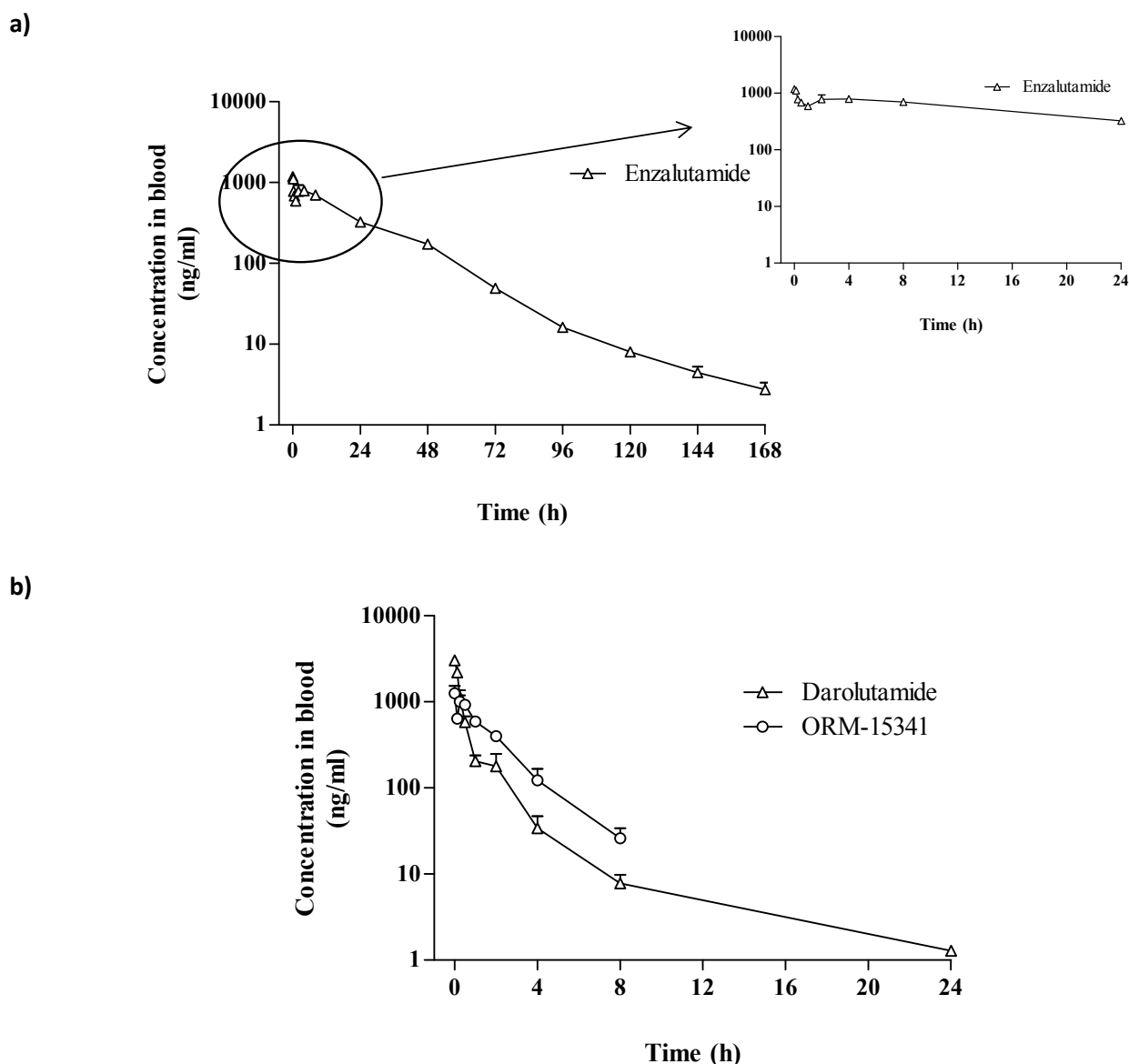
pharmacokinetic parameters are not calculated for *N*-desmethylenzalutamide by using this limited data. Fig. 3b shows mean blood concentration-time profile for darolutamide and ORM-15341, both were quantified till 24 and 8 h, respectively post-dosing by intravenous route. Summary of the pharmacokinetic profiles of enzalutamide, darolutamide and ORM-15341 after single dose intravenous administration in male BalbC mice were shown in Table 6. In order to validate this method the pharmacokinetics results of enzalutamide, darolutamide and ORM-15341 in the present study were compared with earlier reported pharmacokinetic parameters determined using plasma as a matrix [19] and found that good correlation between both measurements. This shows that DBS can be used as a promising alternative suitable to predict exposure of enzalutamide, darolutamide and ORM-15341. *N*-desmethylenzalutamide pharmacokinetic parameters were not calculated due to low exposure and in our earlier study we did not calculate *N*-desmethylenzalutamide pharmacokinetic parameters [19].

**Table 5.** Impact of hematocrit value of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 on the quality control sample concentration (n = 3)

	Concentration spiked	2.80 ng/mL			1493 ng/mL		
	Hematocrit (%)	25	35	45	25	35	45
Concentration found (ng/mL)	Enzalutamide	2.63	2.53	2.79	150	1561	1526
	<i>N</i> -Desmethylenzalutamide	2.96	3.04	2.78	155	1498	1585
	Darolutamide	2.80	2.85	2.86	153	1633	1583
	ORM-15341	3.07	3.06	2.56	146	1604	1498
Accuracy (%RE)	Enzalutamide	0.94	0.90	1.00	1.01	1.05	1.02
	<i>N</i> -Desmethylenzalutamide	1.06	1.09	0.99	1.04	1.00	1.06
	Darolutamide	1.00	1.02	1.02	1.03	1.09	1.06
	ORM-15341	1.10	1.09	0.91	0.98	1.07	1.00
Precision (% RSD)	Enzalutamide	3.96	2.38	5.89	10.2	7.10	9.85
	<i>N</i> -Desmethylenzalutamide	7.12	7.33	8.65	5.87	10.6	8.36
	Darolutamide	9.21	7.12	2.35	11.2	12.3	10.9
	ORM-15341	8.18	5.55	9.38	12.4	2.95	12.3

**Table 6.** Pharmacokinetic parameters for enzalutamide, darolutamide and ORM-15341 following cassette dose *intravenous* administration to mice at 1 mg/kg

PK parameters	Enzalutamide	Darolutamide	ORM-15341
$AUC_{(0-\infty)}$ (ng × h/mL)	24332	1514	2214
$C_0$ (ng/mL)	1180	3042	1251
$T_{1/2}$ (h)	31.0	4.64	1.56
CL (mL/min/kg)	0.68	11.0	---
$V_d$ (L/kg)	1.84	4.42	---



**Figure 3.** Mean blood concentration-time profile of **(a)** enzalutamide in mice blood following *intravenous* cassette dosing of enzalutamide and darolutamide to mice at 1.0 mg/kg; **(b)** darolutamide and ORM-15341 (released from darolutamide) in mice blood following *intravenous* cassette dosing of enzalutamide and darolutamide to mice at 1.0 mg/kg.

## Discussion

The objective of the present method was to develop and validate a new simple, rapid and sensitive DBS method for simultaneous quantification of enzalutamide, *N*-desemthylenzalutamide, darolutamide and ORM-15341 in mice blood and apply to a pharmacokinetic study in mice. DBS [Whatman DMPK FTA or AutoCollect DBS (Ahlstrom-Munksjo) cards] is increasingly becoming an important tool in preclinical drug development, pharmacokinetic/toxicity studies, neonatal screening, clinical pharmacology, forensic toxicology, doping analysis and therapeutic drug monitoring. DBS method offers several advantages like reduced blood/plasma/serum volume per time point, minimally invasive, ease and safety in handling, reduce risk of infection when compared with conventional wet sampling. It offers potential cost saving in storage, shipment, sample processing time (usually done at room temperature) and increase in throughput etc [28,29].

To achieve a DBS method for the first-time for second generation non-steroidal anti-androgens namely enzalutamide and darolutamide along with its active metabolite (*N*-desmethylenzalutamide and ORM-15341, respectively) during method development different options were evaluated to optimize DBS sample extraction and chromatography.

Recovery was very poor (<8 %) with organic solvents (TBME, ethyl acetate, methanol and acetonitrile) for all the analytes. Then combination of methanol/acetonitrile:water in various ratios were systematically explored. The samples extracted with acetonitrile:water (80:20; 50:50 and 20:80, v/v) have shown low recovery (~12 %). At 80:20, 50:50 and 20:80 methanol:water v/v ratios the recoveries ranged between 22-32 % but not reproducible for all the analytes. Water as an extraction solvent gave good recovery for the analytes (~47-50 % for darolutamide and >77-84 % for rest of the analytes) and data was consistent and reproducible.

In order to increase the assay precision, limit the variability between analyte(s) and the IS, to mimic the analyte during ionization, extraction and chromatography we have used apalutamide-*d*<sub>3</sub> as an IS. To avoid the potentially co-eluting peaks from whole blood, which will influence the ionization efficiency of the analytes and the IS, the mobile phase was optimized. The attained LLOQ (0.93 ng/mL) for each analyte (comparable LLOQ attained with plasma earlier reported by us) was sufficient to quantify pharmacokinetic parameters for enzalutamide, darolutamide and ORM-15341 in a pharmacokinetic study in mice. The acceptable limit for both intra- and inter-day accuracy and precision is  $\pm 15$  % of the nominal values for all, except for LLOQ QC which should be within  $\pm 20$ %. In this method, both intra- and inter-day accuracy and precision are well within this limit, indicating that the developed method is precise and accurate for the quantification of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341.

## Conclusions

We developed and validated an analytical method for the determination of enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 in mice DBS samples for these second generation non-steroidal anti-androgens. The method is selective, linear, accurate and precise in the range of 0.93-2000 ng/mL. The DBS samples were stable in the studied conditions and haematocrit did not influence enzalutamide, *N*-desmethylenzalutamide, darolutamide and ORM-15341 quantification. The developed DBS method has several advantages viz., low blood volume (25  $\mu$ L), reduce the large number of animal usage, allows serial blood sampling from mice, useful in toxicokinetic studies etc. Further, blood samples having hematocrit values between 25 and 45 % demonstrated acceptable accuracy and precision in the quantitative measurements. The utility of this assay was demonstrated through its successful application to a mice pharmacokinetic study.

**Conflict of interest:** The authors wish to declare that there are no conflicts of interests in the contents of the manuscript.

## References

- [1] R.L. Siegel, K.D. Miller, A. Jemal. Cancer Statistics 2017. *CA: a Cancer Journal for Clinicians* **67** (2017) 7-30.
- [2] C.J. Ryan, M.L. Cheng. Abiraterone acetate for the treatment of prostate cancer. *Expert Opinion on Pharmacotherapy* **14** (2013) 91-96.

- [3] H.I. Scher, C.L. Sawyers. Biology of progressive, castration-resistant prostate cancer: directed therapies targeting the androgen-receptor signaling axis. *Journal of Clinical Oncology* **23** (2005) 8253-8261.
- [4] L.J. Scott. Abiraterone acetate: A review in metastatic castration-resistant prostate cancer. *Drugs* **77** (2017) 1565-1576.
- [5] A. Mullard. 2012 FDA drug approvals. *Nature Reviews Drug Discovery* **12** (2013) 87-90.
- [6] Z.T. Al-Salama. Apalutamide: First Global Approval. *Drugs* **78** (2018) 699-705.
- [7] A.M. Moilanen, R. Riikonen, R. Oksala, L. Ravanti, E. Aho, G. Wohlfahrt, P.S. Nykänen, O.P. Törmäkangas, J.J. Palvimo, P.J. Kallio. Discovery of ODM-201, a new-generation androgen receptor inhibitor targeting resistance mechanisms to androgen signaling-directed prostate cancer therapies. *Scientific Reports* **5** (2017) 12007.
- [8] J. Semenas, N. Dizeyi, J.L. Persson. Enzalutamide as a second generation antiandrogen for treatment of advanced prostate cancer. *Drug Design Development and Therapy* **7** (2013) 875-881.
- [9] J.A. Gibbons, T. Ouatas, W. Krauwinkel, Y. Ohtsu, J.S. van der Walt, V. Beddo, M. de Vries, J. Mordenti. Clinical pharmacokinetic studies of enzalutamide. *Clinical Pharmacokinetics* **54** (2015) 1043-1055.
- [10] J. Weiss, J. Kocher, C. Mueller, S. Rosenzweig, D. Theile. Impact of enzalutamide and its main metabolite *N*-desmethylenzalutamide on pharmacokinetically important drug metabolizing enzymes and drug transporters. *Biopharmaceutics & Drug Disposition* **38** (2017) 517-525.
- [11] K. Fizazi, C. Massard, N.D. James. ODM-201, a new generation androgen receptor inhibitor for castration-resistant prostate cancer: preclinical and phase I data. *American Society of Clinical Oncology Journal* **31** (suppl 6) (2013) Abstract 65.
- [12] A. Moilanen, R. Riikonen, R. Oksala. ODM-201 - new generation antiandrogen with excellent antiandrogenic and antitumor activity in nonclinical models of CRPC. *European Journal of Cancer* **49** (suppl 2) (2013) Abstract 685.
- [13] Orion Pharma Clinical Study Code: 3104001/EN3386-201 (accessed on 7 June 2018).
- [14] J.H. Song, T.H. Kim, J.W. Jung, N. Kim, S.H. Ahn, S.O. Hwang, N.S. Kang, S.W. Yoo, T.S. Koo. Quantitative determination of enzalutamide, an anti-prostate cancer drug, in rat plasma using liquid chromatography-tandem mass spectrometry, and its application to a pharmacokinetic study. *Biomedical Chromatography* **28** (2014) 1112-1117.
- [15] S. Bennett, J.A. Gibbons, R. Mol, Y. Ohtsu, C. Williard. Validation of a method for quantifying enzalutamide and its major metabolites in human plasma by LC-MS/MS. *Bioanalysis* **6** (2014) 737-744.
- [16] Y. Ohtsu, D.R. Thakker, J.A. Gibbons, K. Tsubota, S. Otsuka, H. Arai. Determination of the androgen receptor inhibitor enzalutamide and its metabolites in animal plasma and brain homogenates using LC-MS/MS and its application to pharmacokinetic studies. *Chromatography* **36** (2015) 115-122.
- [17] K.P. Kim, R.A. Parise, J.L. Holleran, L.D. Lewis, L. Appleman, N. van Erp, M.J. Morris, J.H. Beumer. Simultaneous quantitation of abiraterone, enzalutamide, *N*-desmethylenzalutamide, and bicalutamide in human plasma by LC-MS/MS. *Journal of Pharmaceutical and Biomedical Analysis* **138** (2017) 197-205.
- [18] M. van Nuland, M.J.X. Hillebrand, H. Rosing, J.H.M. Schellens, J.H. Beijnen. Development and validation of an LC-MS/MS method for the simultaneous quantification of abiraterone, enzalutamide, and their major metabolites in human plasma. *Therapeutic Drug Monitoring* **39** (2017) 243-251.
- [19] S.P. Sulochana, N.K. Saini, P. Daram, P.S. Babu, R. Mullangi. Validation of an LC-MS/MS method for simultaneous quantitation of enzalutamide, *N*-desmethylenzalutamide, apalutamide, darolutamide and ORM-15341 in mice plasma and its application to a mice pharmacokinetic study. *Journal of Pharmaceutical and Biomedical Analysis* **156** (218) 170-178.



- [20] M. Herbrink, N. de Vries, H. Rosing, A.D.R. Huitema, B. Nuijen, J.H.M. Schellens, J.H. Beijnen. Development and validation of a liquid chromatography-tandem mass spectrometry analytical method for the therapeutic drug monitoring of eight novel anticancer drugs. *Biomedical Chromatography* **32** (2017) e4147.
- [21] S. Dittakavi, P.K.V.S.P. Nagasuri, S.P. Sulochana, S.M. Saim, S.R. Mallurwar, M. Zainuddin, P. Dewang, S. Rajagopal, R. Mullangi. LC-MS/MS-ESI method for simultaneous quantification of darolutamide and its active metabolite, ORM-15341 in mice plasma and its application to a pharmacokinetic study. *Journal of Pharmaceutical and Biomedical Analysis* **145** (2017) 454-461.
- [22] N. Balaji, S.P. Sulochana, N.K. Saini, A.S. Kumar, R. Mullangi. Validation of a chiral LC-MS/MS-ESI method for the simultaneous quantification of darolutamide diastereomers in mice plasma and its application to a stereoselective pharmacokinetic study in mice. *Biomedical Chromatography* **32** (2018) e4173.
- [23] N. Balaji, S.P. Sulochana, N.K. Saini, A.S. Kumar, R. Mullangi. Validation of a chiral LC-MS/MS-ESI method for the simultaneous quantification of darolutamide diastereomers and ORM-15341 (active metabolite) in mice plasma: application to a pharmacokinetic study in mice. *Drug Research* (2018) in press.
- [24] Y. Xu, E.J. Woolf, N.G. Agrawal, P. Kothare, V. Pucci, K.P. Bateman. Merck's perspective on the implementation of dried blood spot technology in clinical drug development - why, when and how. *Bioanalysis* **5** (2013) 341-350.
- [25] R.K. Rej, R.K. Acharyya, S. Nanda. Asymmetric synthesis of dihydroartemisinic acid through intramolecular Stetter reaction. *Tetrahedron* **72** (2016) 4931-4937.
- [26] X. Pang, Y. Wang, Y. Chen. Design, synthesis, and biological evaluation of deuterated apalutamide with improved pharmacokinetic profiles. *Bioorganic & Medicinal Chemistry Letters* **27** (2017) 2803-2806.
- [27] US DHHS, FDA, CDER, CVM, Guidance for Industry: Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CV), (2018), Rockville, MD, USA.
- [28] P. A. Demirev. Dried blood spots: analysis and applications. *Analytical Chemistry* **85** (2013) 779-789.
- [29] J.D. Freeman, L.M. Rosman, J.D. Ratcliff, P.T. Strickland, D.R. Graham, E.K. Silbergeld. State of the science in dried blood spots. *Clinical Chemistry* **64** (2018) 656-679.